



One-Day (24-26 h) Standardized Laboratory Protocol for Molecular Subtyping of *Campylobacter jejuni* by Pulsed Field Gel Electrophoresis (PFGE)

BIOSAFETY WARNING: Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect plug molds before they are washed. Contaminated items should be disinfected with 10% bleach for at least 30 minutes if they will be washed and reused.

PREPARATION OF PFGE PLUGS FROM AGAR CULTURES

1. Plugs made in reusable plug molds.

Prepare **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)** as follows:

- 10 ml of 1 M Tris, pH 8.0
- 2 ml of 0.5 M EDTA, pH 8.0
- Dilute to 1000 ml with sterile Type 1 water

Prepare 1% SeaKem Gold agarose in **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)** as follows:

- a. Weigh 0.50 g SeaKem Gold (SKG) into 250 ml screw-cap flask.
- b. Add 49.5 ml TE Buffer; swirl gently to disperse agarose.
- c. Remove cap, cover loosely with clear film, and microwave for 30-sec; mix gently and repeat for 10-sec intervals until agarose is completely dissolved.
- d. Place flask in a 55-60°C water bath until ready to use.

* **Note:** The TE Buffer used to make the plug agarose is also used to wash lysed PFGE plugs.

2. Label small tubes (Falcon 2054 tubes or equivalent) with culture numbers.
3. Transfer 2 ml of phosphate-buffered saline (PBS: 0.01 M; pH 7.2; 0.85% NaCl) to small labeled tubes (Falcon 2054 tubes). Use sterile polyester-fiber or cotton swab that has been moistened with sterile PBS to remove some of the growth from agar plate; suspend cells in PBS.
4. Adjust concentration of cell suspensions to:
 - a. Dade Microscan Turbidity Meter: **0.35 - 0.45** (measured in Falcon 2054 tubes). For Falcon tubes 2057 adjust to **0.52 - 0.64**.

- b. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of **0.680** (range of **0.570 to 0.820**).

CASTING PLUGS

1. Add 20 µl of Proteinase K (20 mg/ml stock) to labeled 1.5-ml microcentrifuge tubes (200 µl is needed for 10 cell suspensions.).
2. Transfer 400 µl (0.4 ml) of adjusted cell suspensions to each labeled tube and mix gently with pipet tip. The use of a 1000 µl pipet (P-1000) and tip is recommended for this step; the use of a smaller pipet and tip might cause DNA shearing.
3. Add 400 µl (0.5 ml) melted 1% SeaKem Gold agarose to the 400 µl cell suspension and mix gently by pipeting up and down two to three times. **Over-pipeting could cause DNA shearing.**
4. Immediately, dispense part of mixture into appropriate well(s) of disposable plug mold. Do not allow bubbles to form. Make at least two plugs per specimen. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4°C) for 5 minutes.

LYSIS OF CELLS IN AGAROSE PLUGS

1. **Note:** When 50-ml tubes are used for lysis, two plugs (reusable plug molds) or three plugs (disposable plug molds) of the same strain can be lysed in the same tube.
2. Label 50-ml polypropylene screw-cap or 50-ml Oak Ridge tubes with culture numbers.
3. Calculate the total volume of **Cell Lysis/Proteinase K Buffer** needed as follows:
 - a. 5 ml Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).
 - b. 25 µl Proteinase K stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl). The final concentration of Proteinase K in lysis buffer is 0.1 mg/ml.

Note: Discard any thawed Proteinase K stock solution that was prepared from powder by the user at end of work day. Store commercially prepared Proteinase K solutions according to directions of the supplier.

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.
5. If a flat edge is wanted on the plugs, trim excess agarose from top of plugs with scalpel or razor blade. Open mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tube. Be sure plugs are under buffer and not on side of tube.

Note: The excess agarose, scalpel, spatula, tape, etc. are contaminated. Dispose of, or disinfect them appropriately.

6. **Remove tape from reusable mold.** Place both sections of plug mold, spatulas, and scalpel in 70% isopropanol (IPA) or other suitable disinfectant. **Soak them for 15 minutes before washing them.** Discard disposable plug molds or disinfect them in 10% bleach for 30-60 minutes if they will be washed and reused.

7. Place tubes in rack and incubate in a 50-54⁰C shaker water bath for 15-30 min with constant and vigorous agitation (175-200 rpm). Be sure water level in water bath is above level of lysis buffer in tubes.
8. Pre-heat enough sterile reagent grade water to 50-54⁰C so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

1. Remove tubes from water bath, and carefully pour off lysis buffer. Plugs can be held in tubes with a screened cap (Bio-Rad) or spatula.

Note: Be sure to remove all of the liquid during this and subsequent wash steps by touching lip of tube onto an absorbent paper towel.

2. Add at least 10-15 ml sterile reagent grade water to each tube. Discard water. This acts as a quick wash to remove lysis buffer left behind from the previous step. Add 10-15 ml of sterile reagent grade water that has been pre-heated to 50-54⁰C to each tube and shake the tubes vigorously in a 50-54⁰C water bath for 10-15 min.
 - a. Pre-heat enough sterile **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)** in a 50-54⁰C water bath so that plugs can be washed **three** times with 10-15 ml TE (300-350 ml for 10 tubes) after beginning last water wash.
3. Pour off water, add at least 10 -15 ml pre-heated (50-54⁰C) sterile TE Buffer, and shake the tubes vigorously in 54⁰C water bath for 10-15 min.
4. Pour off TE and repeat wash step with pre-heated TE two more times.
5. Decant last wash and add 5 ml sterile TE (room temperature). Store plugs in 5 ml sterile TE buffer at 4⁰C until ready to do the restriction digestion. Plugs can be transferred to smaller tubes (17-mm x 100-mm, 12-mm x 75-mm, etc.) for storage. Plugs made with disposable plug molds can be stored in 2 ml round bottom tubes containing 1.5 to 1.7 ml of TE.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH *Sma*I

Note: A small slice of the plug or the entire plug can be digested with the primary restriction enzyme; *Sma*I. Restriction digestion of a small slice of the plug is recommended because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. *Kpn*I is recommended as the secondary enzyme for analysis of *Campylobacter jejuni* isolates. The use of a secondary enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable

Note: All PulseNet PFGE protocols use a strain of *Salmonella choleraesuis* ss. *choleraesuis* serotype Braenderup H9812 (ATCC BAA-664) as the molecular size standard. PFGE plugs (or blocks) of the *Salmonella* strain H9812 are made according the "PulseNet One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *E. coli* O157:H7, *Salmonella* serotypes, and *Shigella sonnei* by PFGE" as described in the training manual (www.cdc.gov/pulsenet). This strain is used as a size standard for the normalization and analysis of PFGE patterns for all the organisms tracked by PulseNet, including *E. coli* O157:H7, *Salmonella*, *Shigella*, *Listeria monocytogenes*, and *Campylobacter jejuni*. After plugs of the size standard are made, approximately 2-mm slices are cut and restricted with 40-50 Units of *Xba*I enzyme for 2

hours at 37°C. The plug slices are loaded on the electrophoresis gel in lanes 1, 5, 10 (10-well gel), 1, 5, 10, 15 (15-well gel), or 1, 5, 10, 15, 20 (20-well gel). New lots of *S. Braenderup* H9812 PFGE plugs should be tested with “old” lots to confirm that the pattern and band intensity is the same and that no observable genetic changes have occurred.

***Sma*I Restriction Reactions (*Kpn*I conditions appear in parenthesis)**

1. Label 1.5-ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for the size standard plug slice.
 - a. **Optional Pre-Restriction Incubation Step:** Dilute 10X A buffer (Roche or equivalent) 1:10 with sterile reagent grade water according to the following table.

| Reagent | µl/Plug Slice | µl/10 Plug Slices | µl/15 Plug Slices |
|--|---------------|-------------------|-------------------|
| Sterile Reagent Grade Water | 180 µl | 1800 µl | 2700 µl |
| A Buffer (L Buffer for <i>Kpn</i>I) | 20 µl | 200 µl | 300 µl |
| Total Volume | 200 µl | 2000 µl | 3000 µl |

- c. Add 200 µl diluted 1X A buffer (or L buffer) to labeled 1.5-ml microcentrifuge tubes.
- d. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
- e. Cut a ~2.0 mm-wide slice from test and standard (to be identified) samples with a scalpel (or single edge razor blade, cover slip, etc.) and transfer to tube containing diluted A buffer. Be sure plug slice is under buffer. Replace rest of plug in original tube that contains 5 ml TE buffer. Store at 4°C.

Note: The shape and size of the plug slice that is cut will depend on the size of the comb teeth that are used for casting the gel. Gel wells that are cast with combs that have 10-mm-wide teeth will require a different size plug slice than those cast with combs that have smaller teeth (5.5-mm) teeth. The number of slices that can be cut from the plugs will also depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

- f. Incubate plug slices in 25°C water bath for 5-10 min or at room temperature for 10-15 min.
 - g. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to cut plug slice with pipet tip and that plug slice is not discarded with pipet tip.
2. Dilute 10X A buffer (or L buffer) 1:10 with sterile reagent grade water and add *Sma*I (or *Kpn*I) restriction enzyme (40U/sample) according to the following table (for Roche reagents). Forty units of *Sma*I per plug slice are recommended if restriction is to be carried out in 2 hours. Mix in the same tube that was used for the diluted A buffer.

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20°C) at all times.

| Reagent | µl/Plug Slice | µl/10 Plug Slices | µl/15 Plug Slices |
|------------------------------------|---------------|-------------------|-------------------|
| Sterile Reagent Grade Water | 179 µl | 1790 µl | 2685 µl |
| A Buffer (or L buffer) | 20 µl | 200 µl | 300 µl |
| Enzyme (40 U/µl) | 1 µl | 10 µl | 15 µl |
| Total Volume | 200 µl | 2000 µl | 3000 µl |

3. Add 200 µl restriction enzyme mixture to each tube. Close tube and mix by tapping gently; be sure plug slices are under enzyme mixture.
4. Incubate sample and control plug slices at 25°C (room temp or water bath/chiller or incubator) for 4 h. **For *KpnI*, incubate for 4-6 hours at 37°C.**
5. If plug slices will be loaded into the wells, cast the electrophoresis gel approximately 45-60 min before restriction digest reaction is finished so it will have time to solidify.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. **Confirm that water bath is equilibrated to 55- 60°C.**
2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

| Reagent | Volume in milliliters (ml) | | | | | |
|---------------------------------|----------------------------|------|------|------|------|------|
| 5X TBE | 200 | 210 | 220 | 230 | 240 | 250 |
| Reagent Grade Water | 1800 | 1890 | 1980 | 2070 | 2160 | 2250 |
| Total Volume of 0.5X TBE | 2000 | 2100 | 2200 | 2300 | 2400 | 2500 |

10X TBE:

| Reagent | Volume in milliliters (ml) |
|---------|----------------------------|
| | |

| | | | | | | |
|---------------------------------|------|------|------|------|------|------|
| 10X TBE | 100 | 105 | 110 | 115 | 120 | 125 |
| Reagent Grade Water | 1900 | 1995 | 2090 | 2185 | 2280 | 2375 |
| Total Volume of 0.5X TBE | 2000 | 2100 | 2200 | 2300 | 2400 | 2500 |

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:
 - a. Weigh appropriate amount of SKG into 500 ml screw-cap flask. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
 - b. Remove cap, cover loosely with clear film, and microwave for 60-sec; mix gently and repeat for 15-sec intervals until agarose is completely dissolved.
 - c. Recap flask and place in 55-60°C water bath.

Mix 1.0 g agarose with 100 ml 0.5X TBE for 14-cm-wide gel form (10 or 15 wells)

Mix 1.5 g agarose with 150 ml 0.5X TBE for 21-cm-wide gel form (15 wells)

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

4. A small volume (2-5 ml) of melted 1% SKG agarose may be needed to fill wells after plugs are loaded. Prepare 50 ml by melting 0.5 g agarose with 50 ml 0.5X TBE in 250 ml screw-cap flask as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times. Microwave for 15-20 sec and mix; repeat for 10-sec intervals until agarose is completely melted. Place in 55-60°C water bath until ready to use. Alternatively, save approximately 5 ml of the melted agarose used to cast the gel in a pre-heated (55-60°C) 50 ml flask and place in 55-60°C water bath until used.

Note: Confirm that gel form is level on leveling table, that **front** of comb holder and teeth face the bottom of gel, and that the comb teeth touch the gel platform.

5. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.
6. Remove plug slices from tubes; put comb on bench top and load plug slices as close to the bottom edge of the teeth as possible. Load the plug slices in the following order:
 - a. Load *Salmonella* serotype Branderup H9812 standards in lanes (teeth) 1, 5, 10 (10-well gel) or in lanes 1, 5, 10, 15 (15-well gel).
 - b. Load samples on remaining teeth of the comb.

Remove excess buffer with tissue. Allow plug slices to air dry on the comb for 5-10 minutes or seal them to the comb with 1% SKG agarose (55-60°C).

7. Position comb in gel form and confirm that the plug slices are correctly aligned on the bottom of the comb teeth, that the lower edge of the plug slice is flush against the black platform, and there are no bubbles.
8. Carefully pour the agarose (cooled to 55-60°C) into the gel form.

9. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.)
10. Turn on cooling module (14⁰C), power supply, and pump (setting of 70 for a flow of 1 liter/minute).
11. Remove comb after gel solidifies for 30-45 minutes.
12. Fill in wells of gel with melted and cooled (55- 60⁰C) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in “Option A” above (**Loading Restricted Plug Slices on the Comb**).
2. Cool melted SKG agarose in 55-60⁰C water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

Note: Confirm that gel form is level on gel-leveling table before pouring gel, that front of comb holder and teeth face bottom of gel, and the bottom of the comb is 2-mm above the surface of the gel platform.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run.)
4. Turn on cooling module (14⁰C), power supply, and pump (setting of 70 for a flow of 1 liter/minute) approximately 30 min before gel is to be run.
5. Remove enzyme/buffer mixture; add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.
6. Remove comb after gel solidifies for at least 30 minutes.
7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.
 - a. Load *Salmonella* serotype Branderup H9812 standards in lanes 1, 5, 10 (10-well gel) or in lanes 1, 5, 10, 15 (15-well gel).
 - b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55- 60⁰C). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

A. **SmaI**: Select following conditions on Chef Mapper for *Campylobacter jejuni*.

Auto Algorithm

50 kb - low MW

400 kb - high MW

Select default values except where noted by pressing "enter".

Change run time to 18 hours

(Default values: Initial switch time = 6.76 s; Final switch time = 35.38 s).

B. **KpnI**: Select following conditions on Chef Mapper for *Campylobacter jejuni*.

Two State

Gradient – 6.0V

Change run time to 18 hours

Included Angle – 120

Initial switch time =5.2 s

Final Switch time =42.3 s

Select default values except where noted by pressing "enter".

Note: The electrophoresis running time recommended above are based on the equipment and reagents used at the CDC. Running times in your laboratory may vary (faster or slower) and should be determined empirically.

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide by diluting 40 μ l of ethidium bromide stock solution (10 mg/ml) with 400 ml of reagent grade water (this volume is for a staining box that is approximately 14-cm x 24-cm; a larger container may require a larger amount of staining solution). Stain gel for 20 - 30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen; the solution can be kept in dark bottle and reused 5 - 6 times before discarding according to your institution's guidelines for hazardous waste or use the destaining bags recommended for disposal of ethidium bromide (**Section 10**).

2. Destain gel in approximately 500 ml reagent grade water for 60 - 90 min; change water every 20 minutes. Capture image on Gel Doc 1000, Gel Doc 2000, or equivalent documentation system. If background interferes with resolution, destain for an additional 30-60 min.

Note: If both a digital image and conventional photograph are wanted, photograph gel first before capturing digital image.

3. Follow directions given with the imaging equipment to save gel image as an *.img or *.lsc file; convert this file to *.tif file for analysis with BioNumerics software program (Additional information is in **Section 11** of the PFGE Manual).
4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L reagent grade water or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min before draining water from chamber.

Please note the following if PFGE results do not have to be available within 24 hours:

1. Plugs can be lysed for longer periods of time (up to 2 hours).
2. The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (15-30 min) and at lower temperatures (37⁰C or room temperature). They can be started on Day 1 and finished the morning of Day 2 after overnight refrigeration of the plugs in TE.
3. The restriction digestion can be done for longer periods of time (4-16 hours). Restrict for at least 4 hours when using 20 units of *Sma*I.

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